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RESEARCH PAPER

Genetic diversity and differentiation of the Dybowski's frog (*Rana dybowskii*) in Northeast China

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Abstract: The genetic diversity and population structure of the Dybowski's frog (*Rana dybowskii*) were investigated by using 11 polymorphic microsatellite loci. Total 75 individuals were sampled from six populations in Lesser Khingan Mountains and Changbai Mountains, China. Results showed that allele number of the 11 microsatellite loci was in the range of 2-10 in all populations, with the mean of 5.6. The average expected heterozygosity (H_E) was 0.572, indicating a moderate polymorphism. The results of genetic differentiation coefficient (F_{ST}) showed that population genetic differentiation was significant between Changbai and Lesser Khingan Mountains (p<0.001). This result was verified further by Nei's genetic distance (D_A) based on UPGMA phylogenetic trees and by AMOVA analysis. In conclusion, the populations distributed in Lesser Khingan Mountains and Changbai Mountain are proposed to be two distinct management units (MUs) for their protection and management.

Keywords: Dybowski's frog (*Rana dybowskii*); genetic diversity; genetic differentiation; management units (MUs); microsatellite

Introduction

Dybowski's frog (*Rana dybowskii*), is an important economic species distributed in Northeast China (Lesser Khingan Mountains and Changbai Mountain), Far East of Russia, Korean peninsula, and Japan (Xie et al. 1999). The taxonomy of this species was disputable in history. It was identified as a subspecies of Chinese brown frog (*Rana chensinensis*) before 1999 (Boring 1945; Orlova et al. 1977; Pope 1931; Stejneger 1925; Wei et al. 1991a). After then, it was proposed to be an independent species according to its difference of distribution range and morphology from the *R. chensinensis* (Xie et al. 1999). This proposal was further proved by the analysis of mitochondria DNA (mtDNA) control region that addressed the significant genetic difference between the two species (Jiang and Zhou 2001; Jiang et al. 2002; Yang et al. 2001).

Meanwhile, it was observed in accumulating studies that the Dybowski's frog in different regions of Northeast China has variations in morphology, cytology, and genetics. The female frogs in Changbai Mountains have a high reproductive capacity and oviduct mass (The oviduct of the frog is described as frog oil in traditional Chinese medicine) (Li et al. 2003). The position of anus of the tadpole in Liaoning lies closer to its head relative to that in Changbai Mountains (Wang et al. 2006). Body size of the adult frogs (both male and female) in Lesser Khingan Mountains is larger relative to the frogs in Changbai Mountains and Liaoning regions. The similar trend was also observed in the body size and body mass of tadpoles (Table 1). A cluster analysis based on a set of morphological characteristics further proved the significant variation between populations in Changbai Mountains and Lesser Khingan Mountains (Ying et al. 2008). Moreover, centromere indexes of the eighth chromosome in frogs in Liaoning differed largely from that of the other regions (Table 1) (Shao et al. 1999). The sequence variation of mitochondrial cytochrome *b* gene was 0.3%–1.8% between Liaoning populations and Heilongjiang populations (Yang et al. 2001). However, genetic distances amongst eight populations in Heilongjiang regions, including Yichun, Shangzhi, Wudihe, Shanhe, Suiyang, Fangzheng, Chaihe, and Dongfanghong, inferred from RAPD was not completely matched with their actual, geographical distances (Xiao et al. 2001). Meanwhile, no significant genetic differentiation was detected in subpopulations in the central Changbai Mountains (Li et al. 2009). These results indicated significant differentiation among populations in Lesser Khingan Mountains, Changbai Mountains and Liaoning region, but not within these three regions. However, genetic markers used in these studies are not sensitive and reliable to address genetic

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differentiation in this case. For example, Cyt *b* gene of mtDNA is commonly used in interspecies studies and not sensitive to detect subpopulation differentiations (Yang et al. 2001). RAPD and RAMP heavily rely on template DNA quality that is not applicable to wild populations due to difficulties in sampling and sample preservation prior to lab experiments, and now nearly is given up in the analysis of genetic diversity and differentiation. Therefore, genetic diversity and genetic structure of the Dybowski's frogs in different regions of Northeast China requires further study by using sensitive and reliable molecular markers.

Microsatellite is a kind of short tandom repeat DNA in the genome with high polymorphism (Sota and Sasabe 2006). PCR based analysis is robust and endurable for low quality samples (Holst-Jensen et al. 2003). It has been used in tests of genetic diversity and population differentiation in numerous studies and its validity for this issue has been proved. Therefore, we carried out a systematic study on the genetic diversity and differentiation of the Dybowski's frog in Northeast China by using microsatellites.

Table 1. Morphological and Chromosomal differences in tadpoles and adult Dybowski's frog from different geographic origins

Distribution	Female Average Length (mm)	Female Average Weight (g)	Male Average Length (mm)	Male Average Mass (g)	Length of Tadpoles (mm)	Width of Tadpoles (mm)	Height at Withers of Tadpoles (mm)	Tail Length of Tadpoles (mm)	Hindlimb Length of Tadpoles (mm)	CTC
Heilongjiang	64.64±2.27 [1]	31.6±5.59 [1]	56.7±4.1 [2]							M [8]
Jilin	>65 [3]	28.3±7.10 [4]	54.2±3.9 [2]	24.70±2.18 [4]	34.63±2.97 [7]	8.27±0.70 [7]	7.39±0.77 [7]	22.37±2.18 [7]	0.94±0.05 [7]	M [9]
Liaoning	>63 [5]	22.6±2.3 [6]	>51 [5]	19.4±2.0 [6]	20.85±0.93 [7]	5.41±0.34 [7]	4.48±0.43 [7]	12.8±0.75 [7]	0.63±0.03 [7]	St [10]

[1] (Zhang et al. 2008), [2] (Xie et al. 1999), [3] (Wei et al. 1991b), [4] (Zhao 1982), [5] (Yu et al. 2000), [6] (Tian et al. 2001), [7] (Wang et al. 2006), [8] (Luo; Li 1985), [9] (Wei et al. 1991a), [10] (Shao et al. 1999).

"CTC" means Centromere Type of No. 8 Chromosome "M" means metacentric chromosome; "St" means acrocentric chromosome.

Materials and methods

Sample collection and DNA isolation

A total 75 Dybowski's frogs were captured in from six populations distributing in the Songhua River Basin, the Mudanjiang River Basin, and the Yalu River Basin in Lesser Khingan Mountains and Changbai Mountains (Fig. 1). Frogs were killed by destroying brain and spinal cord. Muscles were isolated from the hind leg of each individual and stored liquid nitrogen immediately and moved to -80°C refrigerator after arrived at lab. Information of samples is shown in Table 2. Genomic DNA was extracted by standard phenol–chloroform protocol (Elphinstone et al. 2003) for all samples, and quantified by using DU-640 nucleic Acid and Protein Analyzer (Beckman USA).

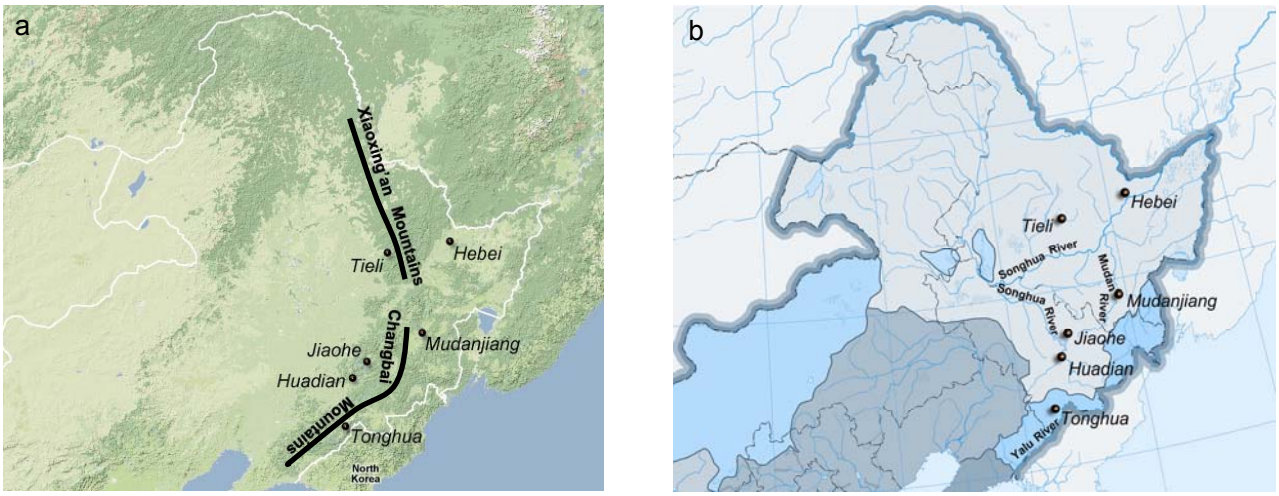

Fig. 1 Sampling sites of Dybowski's frog, (a) Landscape; (b) River system

Microsatellite amplification

Eleven microsatellites of genus *Rana* were selected, including *Rpi100*, *Rpi101*, *Rpi102*, *Rpi104*, *Rpi107* (Hoffman et al. 2003), *RsyC23*, *RsyC52*, *RsyC41*, *RsyD25*, *RsyD40*, *RsyD88* (Julian and King 2003) (Table 3). The core sequences of these microsatellite loci consist of four nucleotide repeat. The 5' end of upper primers of all loci was fluorescently labeled with 5'-FAM, TAMRA, and JOE (Shanghai Invitrogen Bio-Technology Co. Ltd). PCR

reactions were set up in 10-μL system containing 1 × PCR buffer, 0.3 mM dNTP, 2.5 mM $MgCl_2$, 0.3 μM of each of forward and reverse primer, 0.5 units of *Taq* polymerase (TaKaRa, Dalian, China). Cycling was initiated with incubation at 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, appropriate annealing temperature (Table 3) for 30s, 72°C for 30s, and a final extension step of 10 min at 72°C. Then 2 μL of the amplified products were mixed with 20 μL of Hi-Di formamide and 1 μL of internal standard (ILS600, Promega). After denaturation at 95°C for 5 min, they were subjected to capillary electrophoresis on ABI 3130 Genetic Analyzer (Applied Biosystems, USA). Signals were collected and processed using software GENEMAPPER 3.2 (Applied Biosystems, USA). Alleles were defined on the basis of fragment size and the length of core sequence.

Table 2. Summary of information of samples used in this study

Population sampling site	Distribution	River Basin	Sampling year	No. of Samples
Tonghua, Jilin	Changbai Mountains	Yalu River Basin	2006.10	13
Jiaohe, Jilin	Changbai Mountains	Songhua River and a tributary of Mudan River	2006.10	10
Huadian, Jilin	Changbai Mountains	Songhua River Basin	2008.05	12
Mudanjiang, Heilongjiang	Changbai Mountains	Mudan River (main tributary of Songhua River)	2008.05	10
Hebei, Heilongjiang	Xiaoxing'an Mountains	Songhua River Basin	2006.10	15
Tieli, Heilongjiang	Xiaoxing'an Mountains	Songhua River Basin	2006.10	15

Table 3. Primer sequences and annealing temperatures of microsatellite loci used in this study

Locus	Forward 5'-3'	Reverse 5'-3'	Repeat motif	Annealing temperature (ºC)
Rpi100	CACCCTTAAAAGGACAGAACATT	ACCTCTTATTTGTGCCTAACTGAA	$(GATA)_{10}$-$(GATA)_2$-$(GATA)_2$-$(GATA)_2$	63
Rpi101	CGTTAACGCACAGCAAAGGAGTA	GCATGGACAAGGGATGACTTAGAA	$(GATA)_{13}$	58
Rpi102	GTGTGTGTGTTTATTTACTG	CTTCCATTTTAATTGTGT	$(GATA)_{18}$	50
Rpi104	CCTGATAAAGGGGTTTGTGAAT	GAACCATAAAATGTTGGGATAGAG	$(AGAC)_{10}$-$(GATA)_{14}$	50
Rpi107	CCGAGGTACCTAGTTGATGTG	CCGAGGTACCGTGATTATGT	$(GATA)_{16}$-$(GATA)_{10}$-$(GACA)_5$-$(GATA)_{12}$	59
RsyC23	AGGGCATTATTACATTTTGGTC	AGGAAATTACAGAGGACTGTGG	$(TACA)_{10}$	57
RsyC41	GGCAGTCTGGTCCAGTCGTCT	CCACAAAACAGGAATCGGTCATA	$(TACA)_8$	62
RsyC52	CCATACAACCGTGATTACAAAAG	ATATACCACCCTTCCAGAGATG	$(TACA)_{17}$	57
RsyD25	GACCAGAAAGTTATTTCAAGGG	CCCTGTAACTATGTACCAGGAGG	$(TAGA)_{18}$	61
RsyD40	TGATTGATTGTTCACTATTGGG	AAGTAGATTATGTGCTGCAAACTG	$(TAGA)_{19}$	55
RsyD88	TCAATCCATCAGTCTGTCTGTC	GGATTTTGTAAAGAATGCTCCTC	$(TAGA)_{13}$	60

Data analysis

Statistical parameters were calculated by using FSTAT 2.9.3 (Goudet 2001) for 11 microsatellite loci, including number of alleles (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E) and allelic richness (A_R). Fixation index (F_{IS}) of each population was estimated by the combination of FSTAT 2.9.3 and ARLEQUIN 3.01 (Weir and Cockerham 1984; Excoffier et al. 2005). Polymorphism information content (*PIC*) per loci was estimated based on allelic frequency as described in Xing et al. (Xing 2006). The Hardy-Weinberg equilibrium and the heterozygote excess or deficiency of each locus in each population was tested using GENEPOP 3.4 (Rousset and Raymond 1995; Gou and Thompson 1992). Moreover, Linkage disequilibrium per locus was analyzed by Fisher′s test.

Genetic differentiation coefficient (F_{ST}) was calculated to analyze genetic structure and differentiation of populations by using ARLEQUIN 3.01. Significance was tested based on 1,000 replicates random bootstrap. Interpopulation gene flow (N_em) was estimated on the basis of the equation $N_e m=(1-F_{ST})/(4F_{ST})$ (Wright 1965). Nei's genetic distance (D_A; Nei et al. 1983) of each population was calculated using POPULATIONS 1.2 (Olivier Langella 2000). Phylogenetic tree was constructed by using UPGMA clustering, and its accuracy was estimated by the percentage of bootstrapping 1,000 replicates. Genetic differentiation between populations was analyzed by molecular variance (AMOVA) to verify obtained clustering results.

Results and analysis

Genetic diversity

A total 62 alleles were observed on 11 microsatellite loci in 75 samples. Average allele number per loci was 5.6. The allelic number on *RsyD40* (N_A=10) was the highest among 11 microsatellite loci (Table 4), followed by *RsyD88* (N_A=9) and *RsyD25* (N_A=9). *Rpi100* (N_A=2) had the lowest allelic number. One allele unique to "Tieli" population was observed on *RsyC23* and *Rpi104* with frequencies of 0.033 and 0.100, respectively. A unique allele with a frequency of 0.133 was observed on *RsyD40* in "Hebei" population, and on locus *Rpi104* of "Tonghua" population had a unique allele with a frequency of 0.077.

Table 4. Allelic number (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E), allelic richness (A_R), polymorphism information content (*PIC*), Fixation index (F_{IS}) and probability of departure from Hardy-Weinberg Equilibrium of 11 microsatellite loci in each population

Population	*Rpi100*						*Rpi101*					
	N_A	A_R	H_O	H_E	F_{IS}	*PIC*	N_A	A_R	H_O	H_E	F_{IS}	*PIC*
Tonghua	2	1.954	0.154	0.218	-0.044		3	2.723	0.077	0.289	0.657*	
Jiaohe	2	2	0.2	0.377	0.419		3	3	0.1	0.276	0.654	
Huadian	2	2	0.333	0.401	0.154		4	3.957	0.167	0.618	0.735**	
Mudanjiang	2	2	0.3	0.258	-0.125		2	2	0.2	0.182	-0.059	
Hebei	2	2	0.267	0.403	0.349		2	2	0.1	0.335	1.000**	
Tieli	2	1.992	0.267	0.242	-0.12		2	1.897	0	0.136	1.000*	
Mean	2	1.986	0.253	0.317	0.106	0.252	2.67	3.297	0.108	0.306	0.665	0.295
Population	*Rpi102*						*Rpi104*					
	N_A	A_R	H_O	H_E	F_{IS}	*PIC*	N_A	A_R	H_O	H_E	F_{IS}	*PIC*
Tonghua	3	3	0.308	0.738	0.564**		4	3.954	0.539	0.717	0.215	
Jiaohe	3	3	0.2	0.544	0.621*		3	3	0.5	0.61	0.211	
Huadian	2	2	0.25	0.362	0.283		3	2.833	0.333	0.565	0.409	
Mudanjiang	4	4	0.5	0.659	0.274		3	3	0.4	0.664	0.424*	
Hebei	5	4.793	0.267	0.738	0.650**		3	3	0.533	0.661	0.214**	
Tieli	4	3.941	0.267	0.67	0.616**		4	3.969	0.533	0.695	0.261**	
Mean	3.5	4.468	0.299	0.619	0.501	0.628	3.33	3.598	0.473	0.652	0.289	0.607
Population	*Rpi107*						*RsyC23*					
	N_A	A_R	H_O	H_E	F_{IS}	*PIC*	N_A	A_R	H_O	H_E	F_{IS}	*PIC*
Tonghua	3	2.769	0.539	0.428	-0.273		2	2	0.231	0.551	0.556	
Jiaohe	3	3	0.4	0.344	-0.143		2	2	0.4	0.479	0.1	
Huadian	2	2	0.333	0.305	-0.158		2	2	0.417	0.356	-0.222	
Mudanjiang	3	3	0.6	0.468	-0.301		2	2	0.1	0.283	0.64	
Hebei	3	2.637	0.267	0.251	-0.087		4	3.659	0.533	0.552	0.047	
Tieli	3	2.667	0.333	0.42	-0.214		5	4.459	0.333	0.561	0.422	
Mean	2.83	2.505	0.412	0.369	-0.196	0.312	2.83	3.067	0.336	0.464	0.257	0.463
Population	*RsyC52*						*RsyC41*					
	N_A	A_R	H_O	H_E	F_{IS}	*PIC*	N_A	A_R	H_O	H_E	F_{IS}	*PIC*
Tonghua	2	2	0.154	0.428	0.593		6	5.722	0.462	0.877	0.444**	
Jiaohe	2	2	0.2	0.522	0.617		5	5	0.4	0.755	0.486**	
Huadian	3	2.976	0.25	0.378	0.327		4	4	0.25	0.76	0.683**	
Mudanjiang	3	3	0.3	0.4	0.29		5	5	0.1	0.814	1.000**	
Hebei	2	2	0.267	0.394	0.349		6	5.784	0.533	0.793	0.347**	
Tieli	2	2	0.267	0.401	0.349		5	4.333	0.2	0.701	0.726**	
Mean	2.33	2.351	0.24	0.421	0.421	0.379	5.17	5.159	0.323	0.783	0.614	0.744
Population	*RsyD25*						*RsyD40*					
	N_A	A_R	H_O	H_E	F_{IS}	*PIC*	N_A	A_R	H_O	H_E	F_{IS}	*PIC*
Tonghua	5	4.715	0.462	0.646	0.280*		7	6.49	0.539	0.852	0.359**	
Jiaohe	7	7	0.2	0.859	0.772**		7	7	0.3	0.885	0.671**	
Huadian	8	7.643	0.417	0.865	0.540**		6	5.811	0.167	0.808	0.803**	
Mudanjiang	6	6	0.1	0.796	0.882**		6	6	0.1	0.818	0.885**	
Hebei	6	5.879	0.2	0.82	0.767**		7	6.444	0.267	0.809	0.683**	
Tieli	4	3.969	0.333	0.685	0.530**		7	6.584	0.333	0.799	0.600**	
Mean	6	7.381	0.285	0.779	0.629	0.834	6.67	8.154	0.284	0.829	0.667	0.867
Population	*RsyD88*						Mean					
	N_A	A_R	H_O	H_E	F_{IS}	*PIC*	H_O	H_E	F_{IS}	A_R	*PIC*	
Tonghua	4	3.538	0.231	0.662	0.621**		0.336	0.582	0.385			
Jiaohe	5	5	0.3	0.7	0.571**		0.291	0.577	0.498			
Huadian	7	6.891	0.167	0.824	0.805**		0.28	0.567	0.513			
Mudanjiang	6	6	0.1	0.834	1.000**		0.255	0.561	0.598			
Hebei	4	3.992	0	0.729	1.000**		0.294	0.59	0.531			
Tieli	5	4.793	0.267	0.74	0.654**		0.285	0.55	0.498			
Mean	5.17	7.26	0.195	0.748	0.775	0.788	0.291	0.572	0.504	4.475	0.561	

* $0.01<P<0.05$; ** $P<0.01$

The H_o across 11 loci ranged from 0.108 (*Rpi101*) to 0.473 (*Rpi104*), with an average of 0.291. The H_E was between 0.306 (*Rpi101*) and 0.827 (*RsyD40*), averaged 0.572. The averaged A_R and *PIC were* 4.475 and 0.561, respectively (Table 4). H_o was the highest (H_o=0.336) in "Tonghua" population and lowest (H_o= 0.255) in "Mudanjiang" population. However, no significant difference for H_E was observed among six populations (H_E was between 0.550 and 0.590).

Hardy-Weinberg equilibrium and linkage disequilibrium

As estimated by FSTAT 2.9.3, F_{IS}, a measure of the deviation from random mating within the 6 populations, was 0.504, and ranged from -0.301 to 1.000. Among all 11 loci, *Rpi100, Rpi107, RsyC23* and *RsyC52* fit H-W equilibrium (p>0.05, F_{IS} varied from -0.301 to 0.640) in all populations investigated. Locus *Rpi101, Rpi102* and *Rpi104* significantly deviated from H-W equilibrium (p<0.01) in "Huadian", "Hebei", and "Tieli" populations, and the rest four loci (*RsyC41*, *RsyD25*, *RsyD40* and *RsyD88*) significantly deviated from the equilibrium (p<0.01, average F_{IS}= 0.614, 0.629, 0.667 and 0.775, respectively) in all populations (Table 4).

Totally 55 locus pairs which were randomly combined from 11 microsatellite loci were tested for linkage disequilibrium. All locus pairs but three (*Rpi107-Rpi102, Rpi101 -RsyC41, RsyD25-RsyC41*) did not show significant linkage disequilibrium (p>0.05), accounting for 94.55% of the total pairs.

Population genetic differentiation

Genetic differentiation coefficients (F_{ST}) and gene flows (N_em) among six populations were calculated on the basis of the allelic frequency of 11 microsatellites (Tables 5 and 6). F_{ST} values ranged from 0.040 to 0.178, and N_em values were in the range of 2.57 and 12.35. Significant genetic differentiation occurred between the populations in Lesser Khingan Mountains and Changbai Mountains. Moreover, there was an extremely significant genetic differentiation between "Hebei" and "Tieli" populations in Lesser Khingan Mountains (p<0.001), whereas genetic differentiation among the populations in Changbai Mountains was not significant (p>0.05).

Genetic distance (D_A) -based phylogenetic tree showed that the populations in Lesser Khingan Mountains, namely "Hebei" population and "Tieli" population, fell into one cluster (Table 6 and Fig. 2), with the bootstrap test value of 92%, and the four populations in Changbai Mountains were orderly grouped in a cluster, with the bootstrap test value of 85%. This structure was accordance with the geographic relationships among populations (Fig. 1).

The six populations were divided into two groups on the basis of the above results for AMOVA analysis (Table 7). The results showed that genetic differentiation among populations within the same group and among individuals within one population were both extremely significant (p<0.001). Although there was a small genetic variation (F_{CT}=0.057) between the populations of Lesser Khingan Mountains and Changbai mountains, their genetic differentiation was still at an extremely significant level (p=0.008).

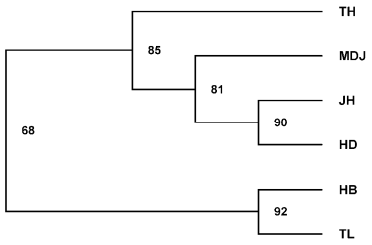

Fig. 2 Phylogenetic (UPGMA) tree of six populations based on Nei's genetic distance D_A

Table 5. Pairwise F_{ST} values (below diagonal) and the probability (above diagonal) of Dybowski's frog among six populations

Population	TH	JH	HD	MDJ	HB	TL
TH		**	***	*	***	***
JH	0.082		NS	NS	***	***
HD	0.125	0.041		NS	***	***
MDJ	0.071	0.040	0.062		***	***
HB	0.131	0.104	0.178	0.158		***
TL	0.129	0.100	0.139	0.132	0.101	

*** P<0.001; ** P<0.01; * P<0.05; NS-not significant

Table 6. Pairwise estimates of gene flow (N_em, below diagonal) and Nei's D_A distance (above diagonal) of the Dybowski's frog among six populations

Population	TH	JH	HD	MDJ	HB	TL
TH		0.144	0.185	0.129	0.184	0.211
JH	5.83		0.685	0.097	0.171	0.166
HD	3.75	12.01		0.132	0.207	0.183
MDJ	6.84	12.35	7.79		0.194	0.180
HB	3.57	4.55	2.57	2.91		0.103
TL	3.64	4.75	3.35	3.55	4.72	

Table 7. Analysis of molecular variance (AMOVA)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation Indices
Among groups	1	83.417	0.201	5.73	0.057**
Among populations within groups	4	95.979	0.263	7.50	0.080***
within populations	542	1652.651	3.049	86.78	0.132***

*** p<0.001; ** 0.001<p<0.01

Discussions and recommendations

Genetic diversity

Genetic diversity is an important genetic parameter for evaluation of evolutionary potential and exploitation potential. Therefore, it is the theoretical basis of conservation strategies and policies. In present study, the average allelic number of 11 microsatellite loci was 5.6, and the average A_R and *PIC* were 4.475

and 0.561, respectively.

The H_E value at species level ranged from 0.306 to 0.827 across 11 loci, with the mean of 0.572. This figure was significantly higher than that in Italian agile frog (*Rana latastei*, H_E, 0.163-0.242) (FicetolaI et al. 2007), moor frog (*Rana arvalis*, H_E, 0.160-0.552) in Sweden and Denmark (Knopp et al. 2007), relict leopard Frog (*Rana onca*, H_E, 0.11-0.71) (Savage and Jaeger 2009), wood frog (*Rana sylvatica*, H_E, 0.14-0.78) (Newman and Squire 2001), Columbia spotted frog (*Rana luteiventris*, H_E, 0.23-0.70) (Funk et al. 2005), and common frog (*Rana temporaria*, H_E, 0.16-0.36) (Matsuba and Merilä 2009), and approximately equal to that in Cascades frog (*Rana cascadae*, H_E, 0.25-0.87) (Monsen and Blouin 2003) and moor frog (*Rana arvalis*, H_E, 0.50-0.68) in the Netherlands (Arens et al. 2007), but slightly lower than that of Chinese wood frog (*Rana chensinensis*, H_E, 0.504-0.855) (Zhan et al. 2009). This demonstrates that the Dybowski's frog in Northeast China has abundant genetic diversity.

Population genetic differentiation

Genetic distance between populations presents genetic variation of a species. The phylogenetic tree (Fig. 2) showed the six populations were clustered to two reliable clusters: one composed of "Heibei" and "Tieli" populations in Lesser Khingan Mountains, the other consisted of four populations in Changbai Mountains. This demonstrated clear differentiation between populations in Lesser Khingan Mountains (bootstrap test value, 92%) and Changbai Mountains (bootstrap test value, 85%). AMOVA analysis showed that genetic variation caused by the geographic difference accounted for 5.73% of total genetic variation. Although the contribution is relatively small, but it caused significant genetic differentiation (p=0.008). This suggested that local populations had evolved unique genetic characteristics, especially between Lesser Kingan Mountains and Changbai Mountains. The strength of gene flow was correlation with the geographic distance among populations. As shown in Table 6, the least gene flow (N_em=2.57) was between HB population in Lesser Kingan Mountains and HD population in Changbai Mountains, while the largest gene flow was between MDJ population and JH population (N_em=12.35) that both of them are belonged to the same water system, the Mudanjiang river system (Table 2). HD population and JH population are the closest in distance, and belonged to Songhua River system (Table 2), therefore, gene flow between them was also strong (N_em=12.01). Such genetic distance agreed with that inferred from morphological features such as body length, fore- or hindleg length and body mass (Xie et al. 1999). These genetic findings are generally consistent with their geographic locations and environmental similarity.

Conservation and management proposition

Management unit is essential for genetic diversity preservation of species. Management unit was usually defined based on the significant difference in allele frequency of nuclear DNA and/or mitochondrial DNA, regardless of the occurrence of systemic differentiation between populations or between distribution regions (Moritz 1994). The results of this study show significant genetic differentiation and weak gene flow between the populations distributing in Lesser Khingan Mountains and Changbai Mountains. Therefore, populations distributed in two regions can be proposed to be two independent management units in term of protection and genetic management. Moreover, unique alleles observed in three populations namely "Tieli", "Hebei", and "Tonghua" suggested that they have unique genetic characteristics. This is in accordance with morphological and physiological traits (Ying et al. 2008). This suggests special attentions should be paid to preserve the genetic uniqueness of these three populations.

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References

Abbott CL, Double MC. 2003. Genetic structure, consevation genetics and evidence of speciation by range expansion in shy and white-capped alba-trosses. *Mol Ecol*, **12**: 2953–2962.

Ardren WR, Borer S, Thrower F, Joyce JE, Kapuscinski AR. 1999. Inheritance of 12 microsatellite loci in *Oncorhynchusmykiss*. *Journal of Heredity*, **90**: 529–536.

Arens P, Sluis Tvd, Westende WPCvt, Vosman B, Vos CC, Smulders MJM. 2007. Genetic population differentiation and connectivity among fragmented Moor frog (*Rana arvalis*) populations in The Netherlands. *Land-scape Ecol*, **22**: 1489–1500.

Boring AM. 1945. Chinese amphibians. Inst. Geobiologie Peking, **13**: 96–100.

Elphinstone MS, Hinten GN, Anderson MJ. 2003. An inexpensive and high-throughput procedure to extract and purify total genomic DNA for population studies. *Mol Ecol Notes*, **3**: 317–320.

Excoffier L, Laval G, Schneider S. 2005. Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evol Bioinform Online*, **1**: 47–50.

FicetolaI GF, Garner TWJ, Bernardi FD. 2007. Genetic diversity, but not hatching success, is jointly affected by postglacial colonization and isolation in the threatened frog, *Rana latastei*. *Mol Ecol*, **16**: 1787–1797.

Funk WC, Blouin MS, Corn PS, Maxell BA, Pilliod DS, Amish S, Allendorf FW. 2005. Population structure of Columbia spotted frogs (*Rana luteiventris*) is strongly affected by the landscape. *Mol Eco*l, **14**: 483–496.

Gou SW, Thompson EA. 1992. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics*, **48**: 361–372.

Goudet J, 2001: FSTAT, version 2.9.3, A program to estimate and test gene diversities and fixation indices. http://www2.unil.ch/popgen/softwares/fstat.htm.

Hoffman EA, Ardren WR, Blouin MS. 2003. Nine polymorphic microsatellite

loci for the northern leopard frog (*Rana pipiens*). *Mol Ecol Notes*, **3**: 115–116.

Holst-Jensen A, Rønning SB, Løvseth A, Berdal KG. 2003. PCR technology for screening and quantification of genetically modified organisms (GMOs). *Analytical and Bioanalytical Chemistry*, **375**: 985–993

Jiang Jianping, Zhou Kaiya. 2001. Phylogenetic relation of Chinese Brown Frogs inferred from mitochondrial DNA sequence of 12SrRNA gene. *Zool Res*, **1**: 27–32.

Jiang Jianping, Xie Feng, Zheng Zhonghua. 2002. Phylogenetic relationships of Chinese Brown Frogs with discussion on the karyotype evolution. *Journal of Si-chuan University (Natural Science Edition)*, **39**: 85–89.

Julian SE, King TL. 2003. Novel tetranucleotide microsatellite DNA markers for the wood frog, *Rana sylvatica*. *Mol Ecol Notes*, **3**: 256–258.

Knopp T, Cano JM, Pierre-André, Merilä CJ. 2007. Contrasting levels of variation in neutral and quantitative genetic loci on island populations of Moor Frogs (*Rana arvalis*). *Conserv Genet*, **8**: 45–56.

Li Yiping, Zhang Jingang, Li Miao, Liu Chunmei, Jiang Dacheng, Deng Minglu. 2003. Study on the origin for oviduetus Ranae. China Journal of Chinese *Materia Medica*, **28**: 16–17.

Li Zhiwei, Yang Baotian, Bai Xiujan. 2009. Genetic diversity of from Changbaishan based on RAMP markers. *Journal of Economic Animal*, **13**: 87–94.

Luo Xueya, Li Jiakun. 1985. Comparative studies on karyotypes of *Rana temporaria chensinensis* from Harbin, Lanzhou and Hongyuan. *Acta Herpetologica Sinica*, **4**: 5–12.

Matsuba C, Merilä J. 2009. Isolation and characterization of 145 polymorphic microsatellite loci for the common frog (*Rana temporaria*). *Mol Ecol Resour*, **9**: 555–562.

Monsen KJ, Blouin MS. 2003. Genetic structure in a montane ranid frog: restricted gene flow and nuclear-mitochondrial disconcordance. *Mol Ecol*, **12**: 3275–3286.

Moritz C. 1994. Defining evolutionarily significant units for conservation. *Trends Ecol Evol*, **9**: 373–375.

Nei M, Tajima F, Tateno Y. 1983. Accuracy of estimated phylogenetic trees from molecular data. II. Gene frequency data. *J Mol Evol*, **19**: 153–170.

Newman RA, Squire T. 2001. Microsatellite variation and fine-scale population structure in the wood frog (*Rana sylvatica*). *Mol Ecol*, **10**: 1087–1100.

Orlova EM, Bakjarev VA, Borkin LJ. 1977. Karyotypes of some brown frogs of Eurasia and a taxonmic analysis of karyotypes of the group. *Proc Zool Inst*, **74**: 81–103.

Paetkau D, Strobeck C. 1995. The molecular basis and evolutionary history of a microsatellite null allele in bears. *Mol Ecol*, **4**: 519–520.

Pope CH. 1931. Notes on amphibians from Fukien, Hainan and other of China. Bull Amer Mus Nat Hist, 61: 78–85.

Rousset F, Raymond M. 1995. Testing heterozygote excess and deficiency. *Genetics*, **140**: 1413–1419.

Savage AE, Jaeger JR. 2009. Isolation and characterization of microsatellite markers in the lowland leopard frog (*Rana yavapaiensis*) and the relict leopard frog (*R. onca*), two declining frogs of the North American desert southwest. *Mol Ecol Resour*, **9**: 199–202.

Shao Yongping, Guo Rui, Xia Qing, Wu Qianhong. 1999. Study on chromosome karyotype and Ag-band of *Rana chensinensis* David from Liaoning Province. *Journal of Fudan University (Natural Sciences)*, **38**: 557–560.

Sota T, Sasabe M. 2006. Utility of nuclear alleIe networks for the analysis of closely related species in the genus carabus, *subgenus ohomopterus*. *Syst Biol*, **55**: 329–344

Stejneger L. 1925. Chinese amphibians and reptiles. *Proceedings of the Na-tional Museum*, **66**: 19–23.

Tian Xinggui, Feng Ying, Liu Yuwen. 2001. Preliminary study on respiration metabolism of *Rana Chensinensis* during hibernation. J*ournal of Xiangfan University*, **22**: 79–82.

Wang Jingqi, Li Pipeng, Lu Yuyan, Dong Bingjun, Zhou Zhengyan, Yu Fenglan. 2006. Description and comparison of tadpoles of *R. huanrenensis* and *R. dybowskii*. *Sichuan Journal of Zoology*, **25**: 349–353.

Wei Gang, Chen Fuguan, Xu Ning, Li Dejun. 1991a. Study on chromosome karyotype and C-banding of *Rana chensinensis kangdingensis* and *Rana chensinensis changbaishanensis*. *Sichuan Journal of Zoology*, **10**: 9–11.

Wei Gang, Chen Fuguan, Xu Ning, Li Dejun. 1991b. New Subspecies of *Rana Chensiensis* from China. *Acta Zool Sin*, **16**: 375–382.

Weir BS, Cockerham CCE. 1984. Estimating F-statistics for the analysis of population structure. *Evolution*, **38**: 1358–1370.

Wright S. 1965. The interpretation of population structure by Fstatistics with special regard to systems of mating. *Evolution*, **19**: 395–420.

Wu Hua, Hu Jie, Wan Qiuhong, Fang Shengguo, Liu Wuhua, Zhang Shuyan. 2008. Microsatellite polymorphisms and population genetic structure of sika deer in China. *Acta Theriologica Sinica*, **28**: 109–116.

Xiao Xiaohong, Zheng Dong, Li Feng, Liu Xuedong. 2001. Population genetic diversity and regional differentiation of Chinese forest frogs (*Rana chensinensis*) in Heilongjiang Province. *Journal of Forestry Research*, **11**: 40–42.

Xie Feng, Ye Changyuan, Fei Liang, Jiang Jianping, Zeng Xiaomao. 1999. Taxonomical studies on brown frogs (Rana) from Northeastern China (Amphibian: Ranidae). *Acta Zool Sin*, **2**: 224–231.

Xing Xiumei. 2006. Studies on molecular genetic diversity of deer or strains in China. Ph.D Dissertation, Chinese Academy of Agricultural Sciences.

Yang Xuegan, Wang Yiquan, Zhou Kaiya, Liu Zhongquan. 2001. Phylogenetic relationships of Chinese Brown Frogs (Rana) based on sequence of mitochondrial cytochrome b gene. *Zool Res*, **5**: 345–350.

Yu Lizhong, Kong Xiangwen, Li Wenchi. 2000. Breeding ecology of Chinese Brown Frog (*Rana chensinensis*). *Journal of Liaoning Forestry Science and Technology*, **2**: 32–34.

Zhan Aibin, Li Cheng, Fu Jinzhong. 2009. Big mountains but small barriers: Population genetic structure of the Chinese wood frog (*Rana chensinensis*) in the Tsinling and Daba Mountain region of northern China. *BMC Genetics*, **10**: 1–10.

Zhang Zhiying, Li Dianwei, Zhao Wenge. 2008. The resting metabolic rate of the aerial respiration of *Rana dybowskii* and *Rana amurensis*. *Chinese Journal of Wildlife*, **29**: 244–247.

Zhao Zhengjie. 1982. Studies on ecology of the *Rana temporaria chensinensis*. J*ournal of Northeast Normal University*, **3**: 89–96.